

Heterogeneous nuclear ribonucleoprotein A1 catalyzes RNA-RNA annealing

(single-stranded nucleic acid binding protein/antisense RNA)

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ABSTRACT Within the nucleus, pre-mRNA molecules are complexed with a set of proteins to form heterogeneous nuclear ribonucleoprotein complexes. A1, an abundant RNA binding protein present in these complexes, has been shown to bind selectively to single-stranded RNAs and destabilize base-pairing interactions. In this study A1 is shown to promote the rate of annealing of complementary RNA strands >300-fold under a wide range of salt concentration and temperature. Maximal annealing is observed under saturating or near saturating concentrations of protein, but annealing decreases sharply at both higher and lower concentrations of A1. Kinetic analysis shows that the rate of annealing is not strictly first or second order with respect to RNA at a ratio of protein/RNA that gives optimal rates of annealing. This result suggests that A1 protein may affect more than one step in the annealing reaction. Two polypeptides representing different domains of A1 were also examined for annealing activity. UP1, a proteolytic fragment that represents the N-terminal two-thirds of A1, displays very limited annealing activity. In contrast, a peptide consisting of 48 amino acid residues from the glycine-rich C-terminal region promotes annealing at a rate almost one-quarter that observed with intact A1. The RNA-RNA annealing activity of A1 may play a role in pre-mRNA splicing and other aspects of nuclear mRNA metabolism.

RNA-RNA base pairing plays a key role in regulating the transfer and expression of genetic information at many levels, including replication, transcription, mRNA processing, and translation (1). Although base pairing of complementary RNAs is sometimes regarded as a spontaneous process, *in vitro* annealing of nucleic acids of the size and complexity of naturally occurring RNA molecules is slow under conditions of ionic strength and temperature approximating those inside most cells (2, 3). *In vivo*, base-pairing interactions may be mediated by proteins that promote or destabilize formation of double-stranded structure.

Recently, several types of proteins have been characterized that unwind or destabilize double-stranded RNA. These include helicases (4, 5), enzymes that deaminate adenosine residues in double-stranded RNA (6, 7), and single-strand-specific RNA binding proteins (8). The function of most such proteins is poorly understood, although helicases are known to be required for the splicing and translation of mRNA (4, 9). Other proteins accelerate the rate of RNA-RNA annealing. These include the Rom (or Rop) protein, which regulates plasmid replication (10), and a small RNA-binding protein found in retroviral cores (11). This laboratory has shown (12) that RNA-RNA annealing proteins are abundant in nuclear extracts active in pre-mRNA splicing. In this report we investigate the ability of an abundant nuclear protein, the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (13,

14), to accelerate the rate of RNA-RNA annealing under a wide range of conditions.

Previous studies have shown that A1, like a number of other DNA and RNA binding proteins known as single-stranded nucleic acid binding proteins (SSBs), binds preferentially to single-stranded nucleic acids (8, 15–17). Under certain conditions SSBs promote denaturation of double-stranded nucleic acids or block annealing of complementary single-stranded sequences. Thus, these proteins are often referred to as helix-destabilizing proteins (8). The helix-destabilizing properties of A1 are associated with the N-terminal two-thirds of the molecule, which has been isolated as a large proteolytic fragment of 195 residues, termed UP1 (15, 18–20). UP1 consists of two copies of a conserved 92-amino acid RNA-binding motif, the RNP consensus domain, which is present in many RNA binding proteins (21, 22). The C-terminal region of A1 constitutes a separate glycine-rich domain that also binds nucleic acids (17, 21, 23).

In vivo, the hnRNP A1 protein is bound to pre-mRNA, where it may play a role in the packaging, processing, and transport of pre-mRNA (13, 14). However, a detailed understanding of its role *in vivo* has proved elusive. The single-stranded binding properties of A1 (16, 17, 24) have been interpreted to suggest that A1 functions to unwind or destabilize intramolecular secondary structure. The ability of A1 to promote efficient annealing suggests that this protein may play a variety of roles in post-transcriptional regulation, facilitating and destabilizing RNA-RNA base pairing.

When a preliminary account of this work was presented in 1990, we learned that Pontius and Berg (25) had independently observed the annealing activity of A1 protein. Their results show that A1 promotes base pairing of DNA and RNA molecules (25). Our work extends this study by examining the kinetics of RNA-RNA annealing in the presence of the intact A1 protein and a short peptide derived from the C-terminal region of A1. The observation of annealing activity associated with this peptide is consistent with a model Pontius and Berg (25) propose for the involvement of the C-terminal region of A1 in annealing. While this report was being revised, similar observations were also reported by Kumar and Wilson (26).

MATERIALS AND METHODS

RNA Transcripts. Synthesis of HB500 and EI200 from plasmids pH500 and pHBS was carried out *in vitro* as described (12). HB500 is a 500-nucleotide (nt) transcript of the human β -globin gene that includes the first exon, first intron, and most of the second exon of the unspliced pre-mRNA. EI200 is a 260-nt RNA that is complementary to 208

Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; nt, nucleotide(s); SSB, single-stranded nucleic acid binding protein.

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nt at the 5' end of HB500. Other complementary transcripts were prepared from the plasmid pHBP, which includes 80 nt from the 3' end of globin exon 2 and 161 nt of pBR322, nt 4010–4170 (27), inserted between opposing phage promoters in pGEM-4 (Promega). One transcript of pHBP, HP250, is 250 nt long and consists of 80 nt of globin sequence followed by 161 nt of pBR322 sequence at its 3' end. The second transcript, P160, is 177 nt long and includes 161 nt of pBR322 sequence complementary to HP250 plus a short noncomplementary region at its 5' end (Fig. 1). Each transcript was uniformly labeled with either ^{32}P - or ^3H -labeled nucleotides and capped at its 5' end by 7-methylguanosine (12).

Proteins and Polypeptides. A1 and two related polypeptides were the generous gift of B. M. Merrill, S. G. Nadler, and K. R. Williams (Yale University). A1 was prepared from a transformed *Escherichia coli* strain overproducing this protein (17). UP1 was prepared by proteolysis of purified recombinant A1 (24). A1(C) is a synthetic peptide corresponding to residues 260–307 of A1 (24). All protein concentrations are based on amino acid analysis.

RNA-RNA Annealing. Annealing assays were carried out in 1 mM MgCl_2 /48 mM NaCl/0.1 mM EDTA/0.5 mM dithiothreitol/2.4% (vol/vol) glycerol/9.6 mM Hepes-KOH, pH 8.0, at 30°C, except where otherwise noted. Assays were initiated by addition of an excess of antisense RNA and protein in rapid sequence. A1 protein solutions contained 0.5 or 2.5 mM dithiothreitol and 0.5 or 1 M NaCl to minimize its tendency to precipitate in the absence of RNA. Two sets of conditions were used for measuring annealing. (i) High concentrations of RNA were used to examine the extent of annealing over 15 min. These reactions included 200–400 nM (nt) ^{32}P -labeled RNA transcript (representing 0.8 nM transcript) and 1.4–2.1 μM (nt) ^3H -labeled complementary RNA (8 nM transcript) and were stopped by addition of RNase T1 (Sigma) at 500 or 1000 units/ml. In experiments in which

annealing was carried out at different temperatures, samples were immediately shifted to 30°C upon addition of RNase. (ii) Low concentrations of RNA were used to study the kinetics of annealing, from 0.17 to 20 min. HB500 RNA (10–80 nM) and EI200 (13–104 nM), representing a 2.5- to 7.5-fold molar excess of the EI200 transcript, were incubated together in 0.125- to 1.2-ml reaction mixtures. Samples of 10–100 μl were removed at appropriate intervals and reactions were stopped either by addition of RNase T1 to 1000 units/ml or by addition of 0.1% SDS plus RNase T1 (1000 units/ml). Samples were incubated for 15 min with RNase T1. Identical samples were removed without RNase treatment to measure input RNA. All samples were treated with proteinase K, extracted with phenol/chloroform, 1:1 (vol/vol), and precipitated with ethanol. For electrophoresis, RNA samples were dissolved in 96% (vol/vol) formamide sample buffer (12), denatured at 95°C, and electrophoresed on 5.5% polyacrylamide/urea gels. Annealing of ^{32}P -labeled RNA was measured by autoradiography, followed by densitometry (Molecular Dynamics model 300A computing densitometer) or liquid scintillation counting of excised RNA bands. RNA annealing was calculated from the size and nucleotide composition of the labeled RNAs. Rates of annealing are given as concentration (nt) of double-stranded RNA formed.

RESULTS

RNA-RNA Annealing Assay. After incubation of ^{32}P -labeled RNAs (HB500 and HP250) with their corresponding ^3H -labeled complements in the presence of A1, RNase digestion produced fragments 210 and 175 nt long (Fig. 1, lanes +A1). The size of these fragments closely matched the size expected for T1 trimming of annealed double-stranded RNAs (Fig. 1 Lower). We found that 55% of HB500 RNA (Left) and 65% of the input HP250 RNA (Right) were annealed. These fragments were not formed in the absence of the complementary RNA (Fig. 1, lanes –EI200 and –P160). The RNase-resistant RNA observed in lane –P160 probably represents formation of T1-resistant intramolecular structure. In the absence of A1, annealing was very slow; 10% or less of the input RNA formed duplex at 15 min (Fig. 1, lanes –A1; cf. Fig. 4A). The combination of RNase digestion and electrophoretic analysis of protected RNA provides a stringent and specific measure of annealing. Digestion of RNA with RNase was very rapid and, when HB500 was used as an annealing substrate, no background was detected in the absence of antisense RNA. Thus, this assay is insensitive to the formation of partially annealed duplexes or adventitious annealing during preparation or analysis of samples.

Requirements for Protein-Catalyzed RNA-RNA Annealing. RNA-RNA annealing was observed over a wide range of A1 concentrations as shown in Fig. 2. Maximal annealing was

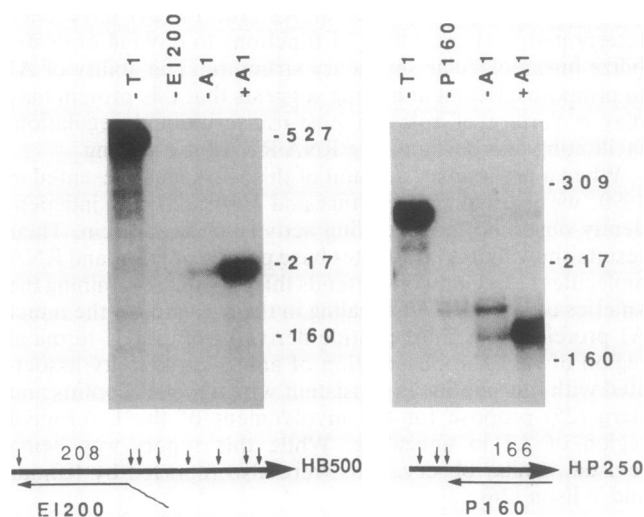


FIG. 1. RNA-RNA annealing activity of hnRNP A1 protein. (Left) Annealing of HB500 and EI200 RNAs. (Right) Annealing of HP250 and P160 RNA. (Lower) Relative position and size (in nt) of the complementary overlap between RNAs. Bold arrows represent ^{32}P -labeled RNAs, thin arrows represent ^3H -labeled RNAs used in the assay, and short vertical arrows represent RNase T1 digestion sites within single-stranded RNA. ^{32}P -labeled transcripts (0.4 μM) were incubated with 10-fold sequence excess of complementary [^3H]RNAs as described for annealing at high RNA concentrations. Input [^{32}P]RNAs are shown in the lanes labeled –T1. Other lanes show RNase-resistant fragments formed in the absence of unlabeled antisense RNA (lanes –EI200 and –P160), in the absence of A1 (lanes –A1), or in the presence of both antisense RNA and A1 (lanes +A1). The concentration of A1 protein was 0.19 μM . The sizes (in nt) of DNA standards are indicated to the right.

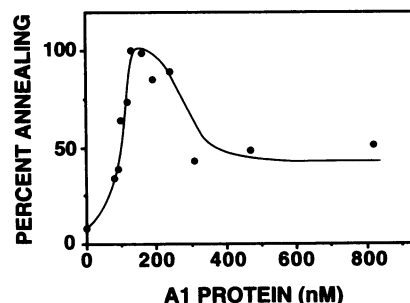


FIG. 2. RNA-RNA annealing at various concentrations of A1. Annealing of 0.4 μM HB500 and 2.1 μM EI200 was carried out as described for annealing at high RNA concentrations but with various concentrations of A1. Maximal (100%) annealing represents protection of 50% of input HB500.

found between 0.13 and 0.24 μM A1 protein, which corresponds to a ratio of 11–19 nt of RNA/A1 molecule. This ratio matches the size of the site required for binding of A1, 12–18 nt at 20–100 mM NaCl (16, 24), suggesting that optimal annealing occurs at concentrations of A1 that saturate or nearly saturate the RNA. The decrease in annealing activity at higher concentrations of A1 varied with different preparations of protein. It appears to reflect a decrease in annealing efficiency at A1 concentrations above saturation, but the reason for this decrease is unclear.

RNA-RNA annealing was also measured at various salt concentrations and at various temperatures to determine conditions for optimal annealing (Fig. 3). Annealing was relatively insensitive to increasing concentrations of MgCl_2 , but a small increase between 0 and 1 mM MgCl_2 was consistently observed (Fig. 3A). Annealing also increased slightly with NaCl concentration between 0.1 and 0.4 M NaCl (Fig. 3B). In the absence of A1, annealing increased with increasing ionic strength as expected (3). Levels of annealing increased with increasing temperature (Fig. 3C) up to at least 42°C. At 45°C, A1 has been reported to begin to denature, as seen by an increase in protein absorbance (24). Uncatalyzed annealing continued to increase with temperature up to 70°C, consistent with a calculated melting temperature of 86°C for double-stranded RNA duplex with this G+C composition (28), and an observed loss of RNase T1 resistance between 80 and 90°C (results not shown). The level of annealing with A1 was not affected by addition of ATP, 0.1–4 mM spermidine, or changes in pH from 6.5 to 8.5 (results not shown).

Kinetics of RNA-RNA Annealing. At the high concentrations of RNA shown above, RNA-RNA annealing in the

presence of saturating concentrations of A1 was very rapid, reaching 80% of maximum levels in 0.5 min or less. To measure the initial rate of annealing, annealing reactions were carried out at lower concentrations of RNA (Fig. 4). These experiments were carried out in 0.2 M NaCl to enhance annealing and reduce possible aggregation of unbound A1 protein. Under these conditions higher protein/RNA ratios were used to obtain optimal annealing. Finally, these reactions were stopped by the addition of SDS to a final concentration of 0.1% to eliminate possible protection of RNA by bound protein. Under these conditions, duplex formation increased rapidly during the first minute, approaching a plateau within 2–5 min. In the absence of A1, <2% of input RNA annealed, representing a 320-fold enhancement of the rate of annealing with A1. The initial rate of annealing increased with increasing concentration of antisense RNA EI200. However, the extent of annealing observed after 10 min varied from 40 to 80% at a concentration of A1 (40 nM) optimized for annealing of the highest RNA concentration. The decrease in the extent of annealing at lower concentra-

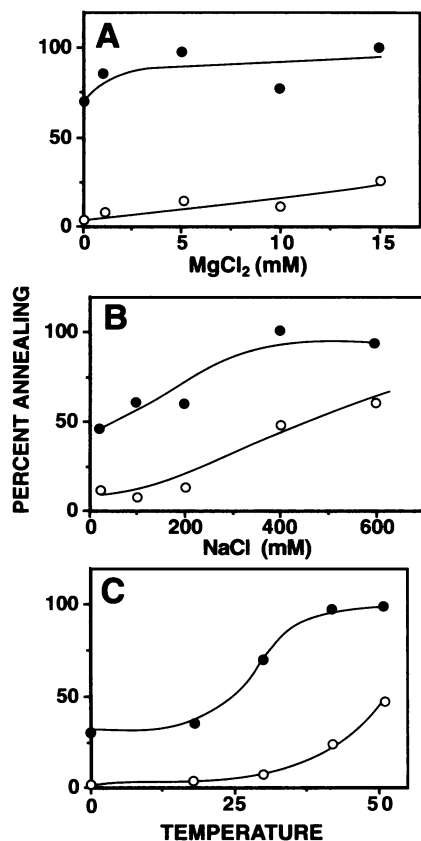


FIG. 3. Requirements for RNA-RNA annealing. Annealing (% maximum) was determined with 0.4 μM HB500 and 2.1 μM EI200 in the presence (●) or absence (○) of 0.19 μM A1. Except as otherwise indicated, annealing reactions were carried out at 30°C in the presence of 48 mM NaCl and 1 mM MgCl_2 as described for annealing at high RNA concentrations.

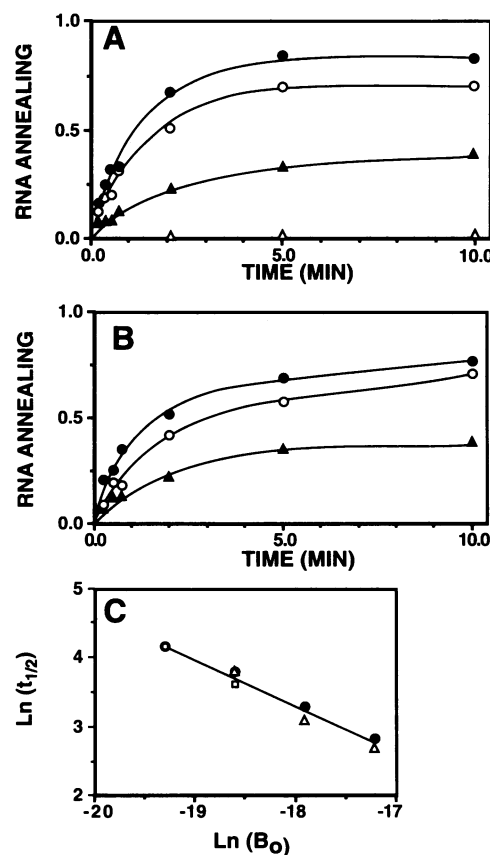


FIG. 4. Kinetics of RNA-RNA annealing. (A) Annealing of HB500 RNA (10 nM) with a 7.5-fold sequence excess of EI200 (39 nM, ●) and 40 nM A1, a 5-fold sequence excess of EI200 (26 nM, ○) and 40 nM A1, or a 2.5-fold sequence excess EI200 (13 nM, ▲) and 40 nM A1. A reaction mixture without A1 containing 10 nM HB500 and a 7.5-fold excess of EI200 (39 nM) was carried out in parallel (△). (B) Annealing of HB500 RNA (10 nM) with a 7.5-fold sequence excess of EI200 and 40 nM A1 (●), a 2.5-fold excess of EI200 and 40 nM A1 (▲), or a 2.5-fold excess of EI200 and 20 nM A1 (○). (C) Annealing reactions carried out with a 2.5-fold sequence excess of EI200 over HB500 and a protein/RNA ratio of 1.7. B_0 represents the nucleotide concentration of HB500 sequences complementary to EI200. Different symbols indicate the results of three experiments (nine points). All reactions (A–C) were carried out in the presence of 200 mM NaCl as described for low RNA concentrations. Reactions in A and B were stopped by addition of SDS followed by RNase, and those in C were stopped by addition of SDS plus RNase.

tions of EI200 was found to reflect the change in the protein/RNA ratio in this experiment. When the concentration of A1 was varied from 10 to 40 nM at a 2.5-fold sequence excess of EI200 over HB500, maximum annealing was observed at 20 nM rather than 40 nM A1 (results not shown). These concentrations of A1 represent the same ratio of protein/RNA. As shown in Fig. 4B, the extent of annealing at 20 nM A1 and a 2.5-fold excess of EI200 approached that observed with 40 nM A1 and a 7.5-fold excess of EI200 after 10 min and was nearly twice that seen at 2.5-fold excess EI200 and 40 nM A1.

The dependence of the initial rate on the concentration of the antisense RNA (Fig. 4A and B) suggests a second-order dependence on RNA concentration. However, the dependence of the final extent of annealing on the protein/RNA ratio complicates interpretation of the kinetic experiments discussed above. Additional experiments were thus performed to examine the relationship between RNA concentration and rate of annealing at constant ratios of protein/RNA and EI200/HB500. At a ratio of A1 to RNA that gave optimal annealing over the range of concentrations tested, a plateau was reached at which 70–80% of input HB500 was annealed. As shown in Fig. 4C, a log-log plot of the half-times of these reactions versus the concentration of the limiting HB500 RNA gave a straight line with a slope of -0.69 . Since a second-order dependence on RNA would give a slope equal to -1 , this result indicates that RNA-RNA annealing is neither strictly first nor second order with respect to RNA under these conditions.

Annealing Activity of A1-Related Polypeptides. The contribution of different domains of A1 to RNA-RNA annealing was investigated by examining annealing activity of two polypeptides, UP1 and A1(C), corresponding, respectively, to the entire N-terminal domain and 48 residues from the glycine-rich C-terminal region of A1 (Fig. 5C). In the presence of UP1 maximal annealing was observed at 0.2–5 μ M protein as shown in Fig. 5A. Maximal annealing was only about one-sixth of that observed with A1 and only 2.5-fold above the level observed without added protein. At concentrations of UP1 $>10 \mu$ M essentially no annealing was seen above that observed in buffer (Fig. 5A) and no further increase in annealing was observed at concentrations of UP1 $<0.2 \mu$ M.

In contrast to results with UP1, substantial annealing was seen with A1(C). Annealing with A1(C), like that with intact A1, fell off sharply at higher and lower concentrations. This decrease was apparent both in the maximal level of annealing observed at high RNA concentrations (Fig. 5A) and in the rate of annealing observed at low RNA concentrations (Fig. 5B). The initial rate of annealing observed in the first 30 s with 62 nM A1(C) peptide was nearly one-fourth that observed for intact A1 in reactions stopped directly with RNase T1 (Fig. 5B). However, subsequent annealing was slower than with A1 and after 10 min only $\approx 50\%$ of input RNA was annealed. These results indicate that the glycine-rich C-terminal region may be directly involved in RNA-RNA annealing by hnRNP A1 protein.

DISCUSSION

Results of this study demonstrate that the hnRNP A1 protein increases the rate of annealing of complementary RNA molecules by >300 -fold relative to that observed in the absence of protein. Efficient annealing is observed in the presence of A1 under a wide range of salt concentrations and temperature. The rate and extent of annealing are critically dependent on the concentration of protein, since annealing decreases at both higher and lower concentrations of A1. Kinetic analysis indicates that the annealing reaction is neither strictly first nor second order with respect to RNA at a constant protein/RNA ratio. One possible interpretation of

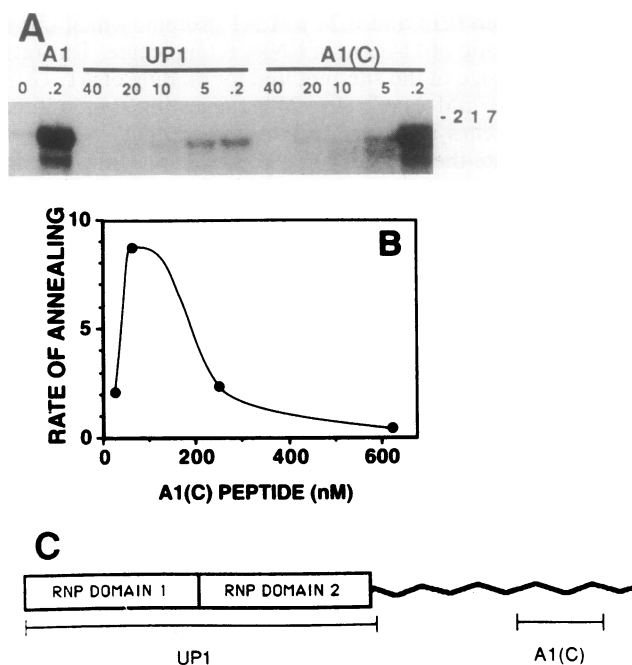


FIG. 5. RNA-RNA annealing activity of A1-related polypeptides. Annealing of HB500 and EI200 was carried out in the presence of UP1 or A1(C) peptide. (A) Reactions were carried out at 0.4 μ M HB500 and 2.1 μ M EI200 as described for annealing at high RNA concentrations. Concentrations of polypeptides in μ M are indicated at the top. Number 217 at the right indicates a DNA size marker. (B) Annealing of HB500 (10 nM) and a 7.5-fold sequence excess of EI200 (39 nM) with the indicated concentrations of A1(C) peptide was carried out as described for annealing at low RNA concentrations. The rate of annealing is expressed as pmol/s. (C) Schematic representation of the primary structure of A1 showing the relative positions of the 92-amino acid RNP consensus domains (boxes), the glycine-rich C-terminal tail (bold lines), and sequences corresponding to UP1 and A1(C).

this result is that both nucleation, a second-order process, and zippering, a first-order process, contribute to the overall rate of annealing. A peptide derived from the C-terminal region of A1 also facilitates annealing. Like A1, the A1(C) peptide shows maximal annealing over a relatively narrow range of protein concentration. Thus these results suggest a relatively complex role for A1 in facilitating RNA-RNA annealing.

In the absence of protein, annealing of complementary nucleic acids occurs by a multistep process in which nucleation is usually rate limiting, and annealing is second order with respect to nucleic acid. For protein-catalyzed reactions, both first- and second-order kinetics have been observed. T4 gene 32 protein and *E. coli* SSB facilitate annealing of DNA molecules with second-order kinetics at saturating concentrations of protein (8, 29, 30). In contrast, RecA protein promotes optimal annealing of single-stranded DNA molecules at subsaturating concentrations of protein with first-order kinetics (31). It has been proposed that T4 gene 32 protein and *E. coli* SSB promote annealing primarily by destabilizing intramolecular structure that can offer a kinetic barrier to formation of fully annealed duplexes (29, 30, 32). The annealing activity of RecA may be attributable to its ability to bind two single-stranded molecules simultaneously (33), thereby increasing the effective concentration of single-stranded molecules. With the exception of the more specialized annealing reaction catalyzed by the Rom protein (10), little is yet known regarding mechanisms of protein catalyzed nucleic acid annealing. The overall kinetics of annealing reflect the relative rates of first- and second-order steps. A

particular protein may affect the rate of more than one step in the pathway. For example, a strictly single-strand-specific RNA binding protein might accelerate nucleation but retard zippering, since the bound SSBs must be displaced during completion of annealing.

Since A1 resembles T4 gene 32 protein and *E. coli* SSB in its single-strand-specific binding to DNA and RNA and has multiple nonidentical binding sites for nucleic acids like RecA, it is possible that several aspects of its interactions with RNA play a role in annealing. Our finding that UP1 lacks substantial annealing activity is consistent with an earlier report that UP1 does not promote DNA-DNA renaturation (18). On the other hand, UP1 has been shown to facilitate renaturation of RNA secondary structure (34). The substantial annealing activity of the A1(C) peptide suggests that the C-terminal region of A1 is important for intermolecular annealing. This domain has been implicated in several interesting properties of A1, including both protein-RNA and protein-protein interactions (23, 24). Both types of interactions may be important for annealing. Despite the involvement of the C-terminal peptide in RNA-RNA annealing, it is likely that the N-terminal domain of A1 also contributes to this process. This region of A1 makes direct contact with RNA (21), and UP1, but not the C-terminal region, displays the single-strand-specific binding characteristic of intact A1 (18, 23).

The experiments reported here demonstrate the ability of A1 to bind RNA molecules in a manner that facilitates base pairing of complementary sequences. This property of A1 may be important *in vivo*. For example, A1 may promote base-pairing interactions between pre-mRNA and small nuclear RNA molecules that are essential for mRNA splicing or 3' end formation (35, 36). Other studies of A1 binding to pre-mRNA *in vitro* have also suggested such a role (37, 38). Since A1 binds tightly to homopolymers and naturally occurring sequences of mixed base composition with relatively little sequence specificity (16, 24, 39), it seems likely that A1 plays a nonspecific accessory role in RNA processing reactions, possibly by facilitating initial interactions between pre-mRNAs and small nuclear RNAs. Another possibility is that A1 may facilitate base pairing between pre-mRNAs and regulatory antisense RNAs. Several examples of naturally occurring antisense RNAs have been described (40–42) that suggest that antisense RNA may act to regulate gene expression in eukaryotes as well as prokaryotes, where antisense regulation is well documented. Finally, A1 may play a role in facilitating the transient formation and destabilization of intramolecular secondary structure *in vivo* and, thereby, modulate the activity of proteins that interact with pre-mRNA in a structure-specific manner. With respect to these possible roles for A1, it is likely that other proteins are also important for RNA-RNA annealing. A number of other hnRNP proteins resemble A1 in sequence and overall structure (43), and a specific splicing factor, SF2, displays RNA-RNA annealing activity *in vitro* (44). Further studies on the annealing activity of A1 are important for understanding both the interactions of this protein with RNA and the mechanism of protein-catalyzed annealing. The wide range of conditions under which A1 promotes annealing also suggests that this protein may prove useful as a reagent for catalyzing or modulating annealing *in vitro*, especially in procedures in which it is desirable to avoid high temperatures, denaturants, or prolonged incubations.

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